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Developing species-specific primers to identify *Bulinus truncatus* and *Bulinus beccari*, the intermediate hosts of *Schistosoma haematobium* in Saudi Arabia

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ABSTRACT

This work aimed to determine the inter- and intra-specific variations in populations of *Bulinus truncatus* and *Bulinus beccari*, the intermediate hosts of *Schistosoma haematobium* in Saudi Arabia, and to develop species-specific primers to identify these snails as a first step in the development of multiplex PCR for simultaneously identifying the snails and diagnosing its infections in a single step. Two populations of *B. truncatus* were collected from Asser and Bisha (A and B), and two *B. beccari* populations were collected from Mahial Asser and Merba (C and D). The snails' genomic DNA was extracted and amplified using 5 different primers. The primers displayed variable intra- and inter-specific differences across the populations. The largest RAPD-PCR fragments were cloned into a vector as a preparatory step for sequencing. Similarity searches for the sequenced cloned inserts revealed no similar sequences in the GenBank database or its associated databases. Specific primers used to target the *B. truncatus* and *B. beccari* genomes were designed using the Gene Runner program and based on the DNA sequences obtained from RAPD fragment sequence analyses. Using these primers for specific PCRs resulted in expected single-band PCR products of 536 bp for *B. beccari* and 478 bp for *B. truncatus*. These results will be helpful for simultaneously identifying *B. truncatus* and *B. beccari* snails and diagnosing *S. haematobium* infections within the snails using single step multiplex PCR.

1. Introduction

According to the Statistical Year Book of Ministry of the Health in Saudi Arabia (2008), the prevalence rate of bilharziasis in the Kingdom of Saudi Arabia (KSA) was 2.78/100,000; 24.6% of the population suffered from urinary schistosomiasis, 75% had intestinal schistosomiasis, and coinfections were found in 0.4% of the population. A total of 55.5% of the schistosomiasis cases were discovered in Saudi individuals compared to 44.5% in non-Saudi individuals. In the geographical regions of the KSA, urinary schistosomiasis has been recorded in Jazan and Asser, intestinal schistosomiasis has been recorded in Ta'if, Al-Bahah, Asser and Bishah, and co-infections have been recorded in Asser.

The snail *Biomphalaria arabica* acts as the intermediate host for *Schistosoma mansoni* in Saudi Arabia (Arfaa, 1976). For *S. haematobium*, three species of snails, *Bulinus truncatus*, *Bulinus beccari* and *Bulinus wrighti*, have been identified as intermediate hosts (Arfaa et al., 1989). However, despite the importance of *B. truncatus*, *B. beccari* and *B. wrighti* in the epidemiology of urinary schistosomiasis in Saudi Arabia, their genetic structures have not yet been elucidated.

Studies investigating the population structure of schistosome-transmitting snails have provided important information regarding the occurrence and distribution of schistosomiasis in various regions. Genetic markers obtained using randomly amplified polymorphic DNA (RAPD) have been widely used in studies on the intra- and inter-population genetic variability of several organisms (Williams et al., 1990; Oliveira et al., 2008, 2010). RAPD has been used in studies on the genetic variability of mollusk populations (Calienes et al., 2004).

For *Bulinus* spp. the RAPD technique was used by various authors to investigate the relationships among different taxa and to confirm the identification of species. Davies et al. (1999) compared the genetic population structures of the freshwater snail *B. globosus* and its trematode parasite *S. haematobium* from 8 river sites in the Zimbabwean highveld by using randomly amplified DNA (RAPD)

Abbreviations: *B. truncatus*, *Bulinus truncatus*; *B. beccari*, *Bulinus beccari*; KSA, Kingdom of Saudi Arabia; PCR, polymers chain reaction; RAPD, randomly amplified polymorphic; RFLP, restriction fragment length polymorphism; *S. haematobium*, *Schistosoma haematobium*; *S. japonicum*, *Schistosom japonicum*; *S. mansoni*, *Schistosom mansoni*.

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markers. Raahauge and Kristensen (2000) characterized the *B. africanus* group species morphologically, anatomically and molecularly by amplification of ITS region, PCR-RFLP and RAPD-PCR. Jones et al. (2001) used RAPD-PCR to investigate the relationships among *B. forskalii* group which is one of the four *Bulinus* species responsible for the transmission of schistosomes in Africa and in the adjacent regions.

Development of species-specific primer is an important step in the establishment of multiplex PCR reaction for detection of parasites within its intermediate host. Vidigal et al. (2002) designed specific PCR primers for Brazilian snail hosts of *S. mansoni* from available sequences of internal transcribed spacer 2 (ITS2) of the ribosomal RNA gene. Jannotti-Passos et al. (2006) developed species-specific primers directed to Brazilian *Biomphalaria* spp. Those primers were used simultaneously in a single multiplex-PCR reaction, and template DNA was obtained from *S. mansoni*-infected and non-infected snails. Kaset et al. (2010) collected Lymnaeidae—the intermediate host of *Fasciola* spp.—from different localities across Thailand and analyzed their 16S rDNA sequences as a molecular signature for classification. Based on the obtained 16S rDNA data three primer pairs were designed that allowed rapid identification of these snail species by PCR. To determine their infection status, PCR primers for *F. gigantica* cathepsin L were used in parallel with the snail 16S rDNA species-specific primers in multiplex PCR analyses. Up to our knowledge species-specific primers for *B. truncatus* or *B. beccari* have been not previously developed.

This work aimed to determine inter- and intra-specific variations in populations of *B. truncatus* and *B. beccari* snails and to develop species-specific primers to identify these snails as the first step in simultaneously identifying these snails and diagnosing *S. haematobium* infections using multiplex PCR.

2. Materials and methods

2.1. Snails

Two populations of Saudi *B. truncatus* snails were collected from Asser and Bisha (A and B), and two *B. beccari* populations were collected from Mahial Asser and Merba (C and D).

2.2. DNA extraction

Genomic DNA extractions from Saudi *B. truncatus* and *B. beccari* snails were performed using the DNeasy Blood & Tissue Kit (Qiagen), according to the protocol of Vidigal et al. (2000). The purified genomic DNA was stored at −70 °C until use.

2.3. Randomly amplified polymorphic DNA (RAPD)

RAPD analyses were performed on purified genomic DNA from Saudi *B. truncatus* and *B. beccari* snails. The procedure was performed using the Ready-To-Go RAPD Analysis Kit (GE Healthcare Limited, Amersham Place, Little Chalfont, Buckinghamshire), according to the manufacturer's protocols and following the protocol described by Bin Dajem et al. (2011). Primers used in this study were (P1, 5′-GGT GCG GGA A-3′; P2, 5′-GTT TCG CTCC-3′; P3, 5′-GTA GAC CCG T-3′; P4, 5′-AAC GCG CAAC-3′; P5, 5′-CCC GTC AGC A-3′. After amplification, 10 μl of each PCR product and a 100-base pair DNA ladder (Invitrogen) were separated on a 1.5% agarose gel, and the banding pattern of the randomly amplified DNA was visualized using ethidium bromide (0.5 μg/ml). The gel was imaged to determine the number and sizes of the separated bands using Gel Pro software (ver. 3.0, USA, 1998) and a Media Sci Image Denistometer 700 (Bio-Rad).

2.4. Similarity coefficient

The genetic variability in the Saudi *B. truncatus* and *B. beccari* populations was evaluated by analyzing the electrophoretic band patterns of RAPD-PCR and determining the similarity coefficient, as described by Dice (1945).

2.5. Purification of the PCR products

The largest and denser PCR resulted from snail populations were gel purified using QIAquick Gel Extraction Kit (Qiagen) according to the manufacturer's recommended protocol. The purified PCR products were stored at −70 °C until they were used for cloning.

2.6. Cloning of the RAPD fragments

PCR fragment of about 550 bp, resulted from the amplification of *B. beccari* genomic DNA using RAPD primer 1 and PCR fragment of about 530 bp resulted from the amplification of *B. truncatus* genomic DNA using RAPD primer 2 were used for cloning step. The selected purified PCR fragments were cloned using a TA cloning kit (Invitrogen). The purified PCR fragments were ligated into the pCRII-TOPO® cloning vector. Each ligation reaction was used separately to transform one shot competent bacteria. To prepare each ligation reaction, the following components were added in the following order: 2 μl of the pCRII-TOPO® cloning vector, 2 μl of salt solution and 4 μl of the purified PCR fragments. The components were mixed gently and incubated at room temperature for 5 min, and the reaction mixture was stored at −70 °C until use. The recombinant plasmids were extracted using a QIAprep Spin Miniprep Kit (Qiagen Inc., Valencia, CA, USA, cat no. 27104) from at least 10 colonies resulted from each transformation.

2.7. Testing of insert presence

A total of 2 μl of the extracted plasmid was digested using 10 units of EcoR1 (Gibco BRL) at 37 °C for 2 h and analyzed using gel electrophoresis. The recombinant plasmids that contained the insert were isolated and sequenced.

2.8. DNA sequence analyses of the recombinant plasmids and sequence similarity search

DNA sequence analyses of the inserts in the recombinant vectors were performed using the ABI PRISM (model 310) automated sequencer. The sequencing primers were the M13 primer and its reverse primer. The results obtained from the sequence analyses were subjected to a similarity search using standard nucleotide–nucleotide BLAST (http://www.ch.embnet.org/software/bBLAST.html; Altschul et al., 1997).

2.9. Design of the diagnostic primers

Specific primers were designed based on DNA sequences obtained from DNA sequence analysis step to target the *B. truncatus* (GenBank ID: JN228343) and *B. beccari* (GenBank ID: JN242244) genomes using the Gene Runner program. The specific primers for detecting *B. truncatus* and *B. beccari* were designed as follows: *B. truncatus*, GRP3FOR1, CGT CAT TTT CCG CAT TCA TT and GRP3REV1, CGT GGG ATT GTT TTT CTT TCA; *B. beccari*, GRP5FOR1, TTC TTT GAT AAA TAT CCG AAG ATC C, and GRP5REV1, GAG CCC GTC GTT ACT CAT AAA.

In addition to *B. truncatus* and *B. beccari*, the genomic DNA of *B. arabica* snails was included to determine the specificity of the primers. Amplification was performed using a Qiagen® Fast Cycling PCR Kit (Qiagen). Each reaction was performed in a final volume of 25 μl containing 1.0× reaction buffer, 50 ng of genomic DNA,